Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 286 (2005) 225 – 237

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas

Michael A. Hale[a], Hideaki Kagami[a,1], Ling Shi[a], Andrew M. Holland[a,2], Hans-Peter Elsässer[c], Robert E. Hammer[b], Raymond J. MacDonald[a,*]

[a]*Department of Molecular Biology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148, USA*
[b]*Department of Biochemistry and the Howard Hughes Medical Institute, The University of Texas, Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148, USA*
[c]*Department of Cell Biology, University of Marburg, 35033 Marburg, Germany*

Received for publication 10 December 2004, revised 29 June 2005, accepted 1 July 2005
Available online 26 August 2005

Abstract

The homeoprotein PDX1 is expressed throughout pancreatic development and is thought to play important roles at multiple stages. We describe the properties of a tet-off regulatory scheme to manage the expression of *Pdx1* in utero. Cessation of *Pdx1* expression at increasingly later gestational times blocked pancreatic development at progressive and morphologically distinct stages and provided the opportunity to assess the requirement for *Pdx1* at each stage. Embryonic PDX1 is depleted below effective levels within 1 day of the initiation of doxycycline treatment of pregnant mice. We show that PDX1, which is necessary for early pancreatic development, is also required later for the genesis of acinar tissue, the compartment of the pancreas that produces digestive enzymes. Without PDX1, acini do not form; the precursor epithelium continues to grow and branch, creating a truncated ductal tree comprising immature duct-like cells. The bHLH factor PTF1a, a critical regulator of acinar development, is not expressed and cells producing digestive enzymes are rare. This approach should be generally applicable to study the in vivo functions of other developmental regulators with multiple, temporally distinct roles.

Keywords: Pancreas; PDX1; IPF1; Transcription factor; Organogenesis; Tetracycline

Introduction

Pdx1 (*Ipf1*) encodes a HOX-like homeoprotein thought to play critical roles at several stages of embryonic pancreatic development, although those roles are not well understood. Pancreatic development begins with the evagination of a dorsal and a ventral epithelial bud from the foregut endoderm at mid-embryogenesis of the mouse (embryonic days 8.5–9) (Pictet and Rutter, 1972; Slack, 1995). PDX1 is present at the onset of pancreogenesis as the two epithelial buds form (Ohlsson et al., 1993), as the epithelium grows and branches to form the network of tubules composed of precursors to acinar and islet cells, and during the development of both acinar and islet lineages (Guz et al., 1995). PDX1 becomes progressively restricted during the later phase of cellular differentiation until it becomes localized principally to the endocrine cells of the islets of Langerhans in the mature pancreas. In mouse embryos lacking a functional *Pdx1* gene, pancreatic growth and development are arrested shortly after the emergence of the prepancreatic buds (Ahlgren et al., 1997; Jonsson et al., 1994; Offield et al., 1996), thereby preventing the study of

* Corresponding author. Fax: +1 214 648 1915.
E-mail address: raymond.macdonald@UTSouthwestern.edu (R.J. MacDonald).
[1] Current address: Department of Tissue Engineering, Nagoya University School of Medicine, Nagoya 466-8550, Japan.
[2] Current address: Monash Immunology and Stem Cell Laboratories, Monash University Clayton Campus, Melbourne, Australia.

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.07.026

later developmental roles. Neither islets, comprising five major cell types (α, β, δ, ε, PP) that produce polypeptide hormones (principally glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively), nor exocrine tissue, comprising acini (which make the digestive enzymes) and ducts (which produce the fluid that transports the secreted acinar enzymes to the intestine), are formed. To identify other developmental functions of *Pdx1*, a scheme to experimentally manage its expression during embryogenesis is necessary.

Targeted gene inactivation to examine the null phenotype and forced transgenic expression to examine the effects of a constitutively active form can be used to investigate gene function in animals. Both approaches have limitations for developmental control genes, however. For example, when a gene needed for early development is ablated, embryonic or neonatal lethality is nearly certain, making the study of gene function later in development or in the mature animal impossible. Similarly, for a gene such as *Pdx1* with roles at multiple stages during a developmental program, germline inactivation can be informative only up to the time of its first function critical to further development. Tissue-specific gene-knockout strategies using Cre recombinase-based deletion have partly overcome these limitations (Branda and Dymecki, 2004). However, this programmed gene inactivation (or activation) is limited by the number of well-characterized promoters available with relevant spatial and temporal expression properties and by the irreversible nature of the recombination event that precludes gene reactivation.

A potentially useful solution to these limitations allows temporal management of a target gene by the application or withdrawal of innocuous small molecule effectors. Pioneered by Gossen, Bujard and their colleagues with the tetracycline-repressible transactivator, tTA (Furth et al., 1994; Gossen and Bujard, 1992), the options have now expanded greatly (Zhu et al., 2002). This approach is more complicated, however, because it requires the inactivation of both alleles of the endogenous locus and the introduction of a regulatable copy of the gene as well as a gene encoding a conditional transcriptional regulator, such as tTA. The effectiveness of this genetic regulatory strategy in animals often has been incomplete. In this report, we demonstrate an effective variation of the tet-off (Gossen and Bujard, 1992) regulatory scheme to inactivate the expression of *Pdx1* in utero and identify additional developmental periods requiring the presence of PDX1.

To control the transcription of *Pdx1* in mice, we substituted the amino acid coding sequence of the tetracycline-transactivator (tTA-off) for that of the endogenous *Pdx1* locus by homologous recombination and introduced a tTA-responsive transgene encoding PDX1 (Fig. 1 and Holland et al., 2002). The tTA-for-Pdx1 substitution coincidentally places tTA under the control of all the regulatory sequences of the endogenous *Pdx1* locus and abolishes production of PDX1 from that allele. As mice with one active *Pdx1* allele develop normally (Ahlgren et al., 1996; Jonsson et al., 1994; Offield et al., 1996), these mice can be maintained in a heterozygous state. To provide a tetracycline-regulated source of PDX1, transgenic mice were created bearing a bicistronic transgene (tetO-Pdx1) encoding a *Pdx1* minigene and a reporter gene (either nlacZ or EGFP) linked through an IRES and driven by the tTA-responsive promoter of Gossen and Bujard (1992). We previously reported the use of this regulatory scheme to suppress *Pdx1* expression in adult mice (Holland et al., 2002).

In this report, we describe the properties of doxycycline regulation and the effects of *Pdx1* inactivation on exocrine pancreatic development in vivo. We show that inactivation of the *Pdx1* transgene just prior to the initiation of acinar cell differentiation prevents that differentiation.

Methods

Knock-in/out and transgenic mice

Construction of the vectors for the tTA knock-in/out of the *Pdx1* gene by homologous recombination in ES cells and for the EGFP form of the bicistronic tTA-responsive *Pdx1* transgene has been described (Holland et al., 2002). The LacZ form of this *Pdx1* transgene has the β-

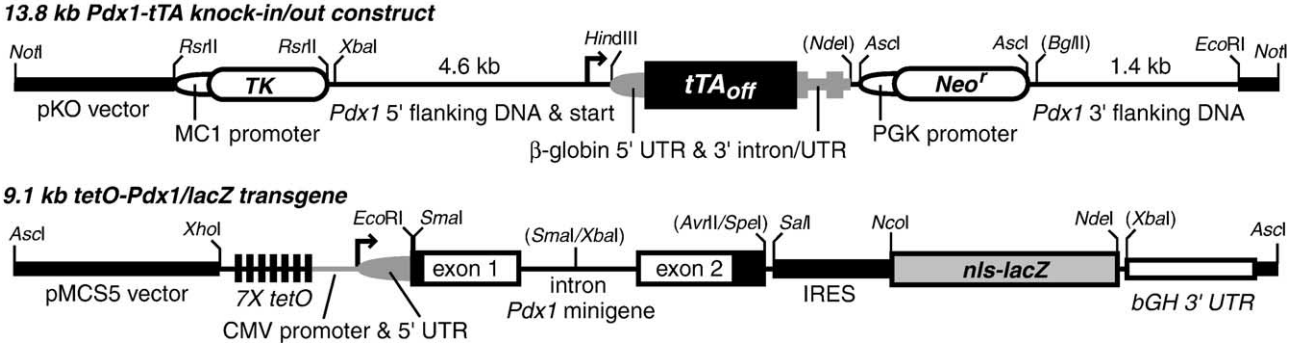

Fig. 1. The designs of the tTA knock-in/out construct for the *Pdx1* gene and the tTA-responsive Pdx1 transgene. The details are discussed in Methods and Results.

galactosidase coding region in place of the *EGFP* gene. Generation of mice bearing the altered (*Pdx1*^{tTA}) allele and creation of the transgenic mice bearing the EGFP form of the bicistronic *Pdx1* transgene were also described previously (Holland et al., 2002). Details of the knock-in/out and transgene constructs, their optimization, and preliminary tests in transfected cells are available on request. The genotypes of mice and embryos were determined by PCR as described (Holland et al., 2002). Treatment of pregnant mice with doxycycline (Sigma, St. Louis, MO) was initiated with a single administration by oral gavage (0.2 ml of a 1 mg/ml solution) and maintained with 1 mg/ml doxycycline in drinking water containing 5% sucrose.

Immunohistochemistry and immunofluorescence

Tissues dissected from embryos and adult mice were fixed in 4% paraformaldehyde for several hours at 4°C, dehydrated using increasing concentrations of ethanol up to 100%, treated with Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI), and embedded in Paraplast Plus Tissue Embedding Medium (Kendall, Mansfield, MA). Five micrometer thick tissue sections were dewaxed by heating at 60°C for 30 min followed by immersion in Clear-Rite 3. Sections were then rehydrated by immersion in decreasing concentrations of ethanol from 100% to 30%, followed by several washes in water. Antigen retrieval was performed using Antigen Unmasking Solution™ (Vector Laboratories Inc., Burlingame, CA) and microwave heating under conditions standardized in our laboratory. After cooling to room temperature, sections were equilibrated by several rinses in PBS and blocked with 10% normal donkey serum (Jackson Research Laboratories, West Grove, PA) in PBS for 1 h at room temperature before the application of primary antibodies.

Primary antibodies were diluted in 5% normal donkey serum and were the following (the dilutions for immunofluorescence (IF), IF with tyramide signal amplification (IF-TSA), and immunohistochemistry (IHC) are noted): rabbit anti-PDX1 (a gift from C.V.E. Wright, Vanderbilt U.) (1:8000 IHC, 1:400 IF), rabbit anti-GLUT2 (a gift from B. Thorens, Lausanne, Switzerland) (1:200 IF, 1:5000 IF-TSA), guinea pig anti-glucagon (Linco, St. Charles, MI) (1:10,000 IF), rabbit anti-amylase (MacDonald et al., 1977) (1:2000 IF), goat anti-carbonic anhydrase II (Santa Cruz, Santa Cruz, CA) (1:100 IF), mouse anti-cytokeratin 7 (Chemicon, Temecula, CA) (1:1000 IF-TSA), rabbit anti-carboxypeptidase A (Chemicon) (1:1000, IF), rabbit anti-HES1 (Tetsuo Sudo, Toray Pharmaceutical Research Lab) (1:1000, IF-TSA), anti-beta-galactosidase (MP Biomedicals) (1:400 IF). Application of primary antibodies was overnight for immunofluorescence and 1 h for IF-TSA at room temperature. Sections were incubated with the secondary antibody for 1 h at room temperature. Secondary antibodies were (with dilutions) FITC-conjugated donkey anti-rabbit IgG (Jackson Research Laboratories, West Grove, PA) (1:200); and Rodamine Red-conjugated donkey anti-rabbit, anti-goat, and anti-guinea pig IgG (Jackson Research Laboratories) (1:200). Tyramide signal amplification was performed with the TSA Kit from Molecular Probes (Eugene, OR). Immunohistochemical detection used peroxidase-conjugated anti-rabbit or anti-guinea pig immunoglobulins. Cover slips were mounted using the Prolong® Antifade Kit (Molecular Probes) mounting medium.

Histochemical staining for β-galactosidase activity

Whole guts dissected from embryos and fetuses were washed twice in cold PBS, and fixed at 4°C in Solution A (100 mM potassium phosphate, pH 7.4, 5 mM EGTA, 2 mM $MgCl_2$) containing 0.2% glutaraldehyde. Tissues were permeabilized by three 10 min washes in Solution A containing 0.02% NP40 and 0.01% 7-deoxycholic acid at room temperature. Tissues were then stained for β-galactosidase activity by incubation in Solution A containing 5 mM each K_3[Fe(CN)6] and K_4[Fe(CN)6] and 0.5 mg/ml X-Gal (Roche, Indianapolis, IN) at room temperature for several hours on a rotating platform. Tissues were postfixed in 4% paraformaldehyde for several hours at 4°C. For sectioning, the fixed tissues were dehydrated using a graded series of ethanol concentrations (70% to 100%) followed by two washes in Clear-Rite 3 and then embedded in Paraplast Plus. Tissue sections were lightly counterstained with Eosin-Y (Richard-Allan Scientific). Wholemount, β-galactosidase-stained embryos and guts for photography were first cleared for several hours in Cedarwood Oil (Polysciences, Inc., Warrington, PA) following the ethanol dehydration steps. Cleared tissues were imaged in several focal planes using an Olympus SZX12 microscope equipped with an Optronics® (Goleta, CA) digital camera and Magnafire camera software and a composite in-focus image was then generated for each tissue using Image-Pro software (MediaCybernetics, Silver Spring, MD).

Microscopy and imaging

Whole en bloc tissues were imaged using a Leica M420 dissection scope. Tissue sections were imaged for hematoxylin and eosin (H&E) staining and for immunofluorescence using a Leica DMRXE microscope. Both microscopes were equipped with a Hamamatsu color chilled 3CCD camera. Images were processed using Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA).

Analysis of the neonatal epithelial remnant by digital volumetric imaging (DVI) was performed by Microscience Group, Inc., as described by Ewald et al. (2002). The 3D image of the epithelial remnant was reconstructed without the need for glass slide-mounted serial sections from the images of 1500 pseudo-H&E-stained sections using RES-View 3.0 volume visualization software.

For electron microscopy, dissected pancreatic remnants were cut into pieces no larger than 1 mm^3 and fixed by

immersion in a solution containing 2.5% glutaraldehyde, 2.5% paraformaldehyde, 0.05% (w/v) picrinic acid in 0.1 M cacodylate buffer (pH 7.35) for 3 h at room temperature, followed by several rinses in 0.1 M cacodylate buffer (pH 7.35). After dehydration and embedding in epon, thin sections were stained with uranyl acetate and lead citrate and examined and photographed with a Zeiss EM109 electron microscope.

RT-PCR

Total cellular RNA was isolated from individual dissected embryonic pancreas with TriZol Reagent (Invitrogen, Carlsbad, CA) and from neonatal and adult pancreas using the guanidine thiocyanate procedure (MacDonald et al., 1987). RNA preparations from age- and genotype-matched embryos were pooled, whereas RNAs from neonatal and adult pancreas remained individual samples. One microgram aliquots of RNA were reverse transcribed using random primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). For quantification, preliminary experiments determined the number of PCR cycles for each mRNA to be within the range for a proportional change in the amount of product for the change of input cDNA. Amplification products were detected by standard Southern hybridization with a gene-specific oligonucleotide probes. The amount of hybridization was measured with a PhosphorImager and quantified using ImageQuant software (Molecular Dynamics). The amount of amplified product from the reactions within the range of proportional response to template was averaged and used to quantify the level of an mRNA relative to the level of the mRNA for actin.

PCR reactions consisted of 25 ng of cDNA template, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.0–1.5 mM $MgCl_2$, 150 ng each oligonucleotide, 0.2 μM each dNTP, and 1 unit Taq DNA polymerase (Roche) in a 50 μl reaction. Amplification conditions, including the PCR primer pairs and Southern hybridization probes, for the mRNAs of actin, the endogenous *Pdx1*, the transgenic *Pdx1*, tTA, amylase, β-galactosidase, Ptf1a, carboxypeptidase A, and TBP are available on request.

Results

tTA-mediated activation of the reporter transgenes in mice

At least three conditions must be met to create mice with normal development in which all PDX1 is produced from a regulated transgene: (i) replacement of the *Pdx1* coding region with that of tTA-off must create a *Pdx1*-null phenotype; (ii) expression of tTA in the pancreas cannot be deleterious to pancreatic development or function; and (iii) a tetracycline-regulated, Pdx1 transgene must be able to rescue pancreatic development in $Pdx1^{tTA/tTA}$ mice. The creation of ES cell clones with the tTA-substitution in the *Pdx1* locus, the generation of mice carrying this modification, and the creation of transgenic mice bearing the bicistronic tetO-Pdx1-EGFP transgene have been described (Holland et al., 2002). We generated tetO-Pdx1-nlacZ transgenic mice similarly and emphasize the use of these mice for developmental studies. The Pdx1-tTA knock-in/out construct for homologous recombination and the tTA-regulated Pdx1 transgene were designed to help ensure efficient production of the tTA protein and, in turn, effective levels of PDX1 (Fig. 1). Because a long and highly base-paired 5′ UTR can limit the rate of translational initiation, we removed most of the 5′ UTR of the Pdx1 mRNA in both the knock-in/out and transgenic constructs.

Mouse embryos homozygous for $Pdx1^{tTA}$ do not form a pancreas (Fig. 2B and Holland et al., 2002), consistent with previous descriptions of the effects of *Pdx1* gene inactivation (Jonsson et al., 1994; Offield et al., 1996). For mice with a single $Pdx1^{tTA}$ allele, the formation of the pancreas was normal (Fig. 2A); in adult mice, exocrine and endocrine tissue masses and architecture were normal as well. Adult $Pdx1^{+/tTA}$ mice gradually acquire an impaired endocrine response to glucose challenge (Holland et al., 2002), just as for mice with one allele disrupted by lacZ-insertion (Dutta et al., 1998). Thus, although tTA expression may be deleterious to mammalian cells in certain instances (Saez et al., 1997; Schokett and Schatz, 1996), we detected no effects on pancreatic development or function.

We derived and tested eight independent transgenic lines bearing either nlacZ- or EGFP-forms of the tetO-controlled *Pdx1* for their ability to produce sufficient PDX1 to rescue pancreatic development in homozygous $Pdx1^{tTA/tTA}$ embryos. Each of the eight transgenic founders was mated with $Pdx1^{+/tTA}$ heterozygous mice, the $Pdx1^{+/tTA};Tg^{Pdx1}$ offspring were intercrossed, and the neonates were examined for pancreatic tissue (e.g., Fig. 2C) and genotyped by PCR. The transgenic locus of five of the eight lines restored pancreatic development of $Pdx1^{tTA/tTA}$ embryos to some extent (Table 1). The ability to rescue pancreatic development was not correlated with transgene copy number. Three of the lines, including the Pdx1-nlacZ line 958-1, appeared to rescue most effectively and were analyzed further. Approximately 1 out of 5 pups with the regulated genotype ($Pdx^{tTA/tTA};Tg^{Pdx1}$) from these three lines was born without a pancreas. We do not know the basis for this incomplete rescue penetrance; however, the common observation that long tandem arrays of transgenes are readily silenced (Garrick et al., 1998) suggests that in some embryos the arrays either are not activated or are inactivated during development. The use of multicopy transgenes may not be the optimal strategy. Modification of the other allele of the targeted gene (e.g., *Pdx1*) to make it tetracycline-regulated (e.g., tetO-Pdx1) may be more effective and also would facilitate mating strategies to provide a tetracycline-responsive, rescue genotype (i.e., $Pdx1^{tTA/tetO\text{-}Pdx1}$). Nevertheless, an 80% rescue rate is sufficient for the experimental purposes described in this report.

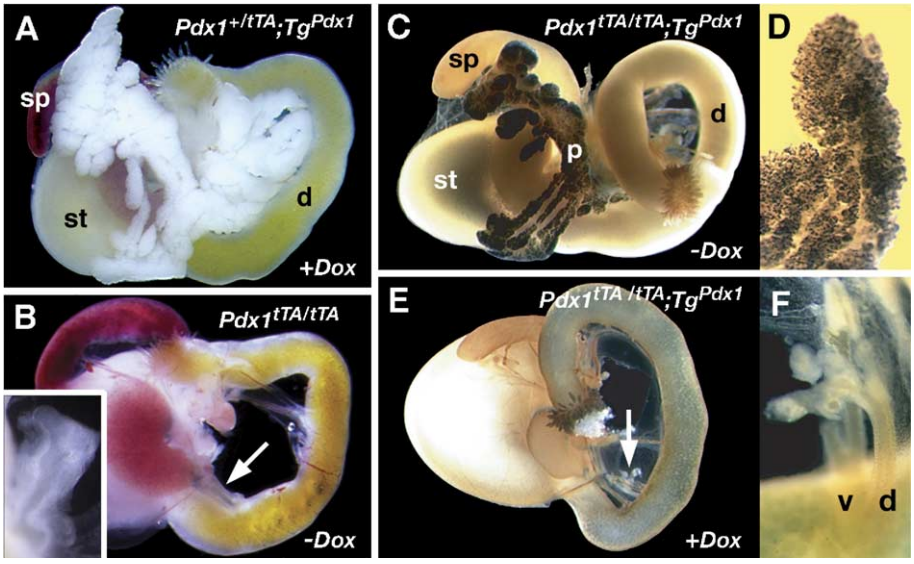

Fig. 2. The Pdx1-nlacZ transgene conditionally rescues pancreatic development of *Pdx1*-deficient (*Pdx1*$^{tTA/tTA}$) embryos. All images are of dissected viscera from postnatal day 1 (P1) pups. (A) Normal pancreatic development of a *Pdx1*$^{+/tTA}$;*Tg*$^{Pdx1\text{-}nlacZ}$ neonate. (B) For *Pdx1*-deficient embryos (*Pdx1*$^{tTA/tTA}$), only a very small pancreatic remnant (*arrow* and inset) forms. (C) A pancreas of nearly full size forms in embryos with the regulated genotype, *Pdx1*$^{tTA/tTA}$;*Tg*$^{Pdx1\text{-}nlacZ}$. The pancreatic tissue stains blue due to the β-galactosidase staining derived from expression of nlacZ on the transgene. (D) Pancreatic nuclei stain for β-galactosidase activity derived from the nlacZ transgene. (E) Neonates of the regulated genotype from mothers treated with doxycycline from the morning of conception are born with small ductal remnants (arrow) derived from the ventral (v) and dorsal (d) pancreatic buds (F), very similar to the pancreatic remnant of pups with the germline *Pdx1* knock-out (B). sp, spleen; st, stomach; d, duodenum; p, pancreas.

For developmental studies, expression of the transgene must coincide closely with that of the endogenous *Pdx1* gene. Of principal concern was the additional intermediate transcription step between activation of the *Pdx1*^{tTA} locus and the PDX1-expressing transgene. Embryonic expression of the tetO-Pdx1 transgene, activated by tTA produced from the *Pdx1*^{tTA} allele, recapitulated the temporal and spatial expression pattern of *Pdx1* (Fig. 3). At E9.5, transgenic β-galactosidase was present in the nascent dorsal and ventral buds. By E11.5, expression of the transgene had spread appropriately to the intervening duodenum, the bile duct and the caudal stomach. All PDX1-positive cells were also β-gal-positive, although the levels of the two proteins were not always in the same ratio (Figs. 3A–C). Subsequent expression marked the branching epithelium precisely. At mid-development (Fig. 3, e14.5 and D), nearly all of the epithelial cells, including those forming acini as well as islet cell clusters, stained for β-galactosidase. At birth, β-galactosidase staining was high in islet cells and low in acinar cells (Fig. 3E), consistent with the maintenance of high *Pdx1* expression selectively in islet β-cells (Guz et al., 1995). Moreover, the

Table 1
Rescue of pancreatic development by tetO-Pdx1 transgenes

Transgene	Line	Copy number	# w/ rescue genotype[a]	Phenotype		
				Rescue	Partial	Apancreatic
Pdx1-nlacZ	957-8	19	2	1	1	0
	958-1	39	30	20	3	7
	958-2	8	3	0	0	3
	958-6	18	2	0	2	0
	958-9	52	1	0	0	1
Pdx1-EGFP	951-1	10	2	0	0	2
	953-1	11	13	6	5	2
	956-6	12	8	2	3	3
Totals	8		61	29 (48%)	14 (23%)	18 (29%)
Total percent rescued		43/61 = 71%				
Three most effective lines		34/43 = 79%				

The ability of eight independent tetO-Pdx1 transgenic lines to rescue pancreatic development of *Pdx1*$^{tTA/tTA}$ embryos. Each line was tested for the ability to rescue pancreatic development in Pdx1$^{tTA/tTA}$ embryos. Neonates were examined for the presence of a pancreas. 'Rescue' was applied to pups that had an estimated 50% or more of normal pancreatic size; 'partial rescue' was between 10 and 50%; apancreatic pups had no discernible pancreas. The number of transgene copies for each line was determined by quantitative dot hybridization of genomic DNA with either EGFP or nlacZ probes.

[a] Total number of newborn pups with the rescue genotype (*Pdx*$^{tTA/tTA}$;*Tg*^{Pdx1}) tested.

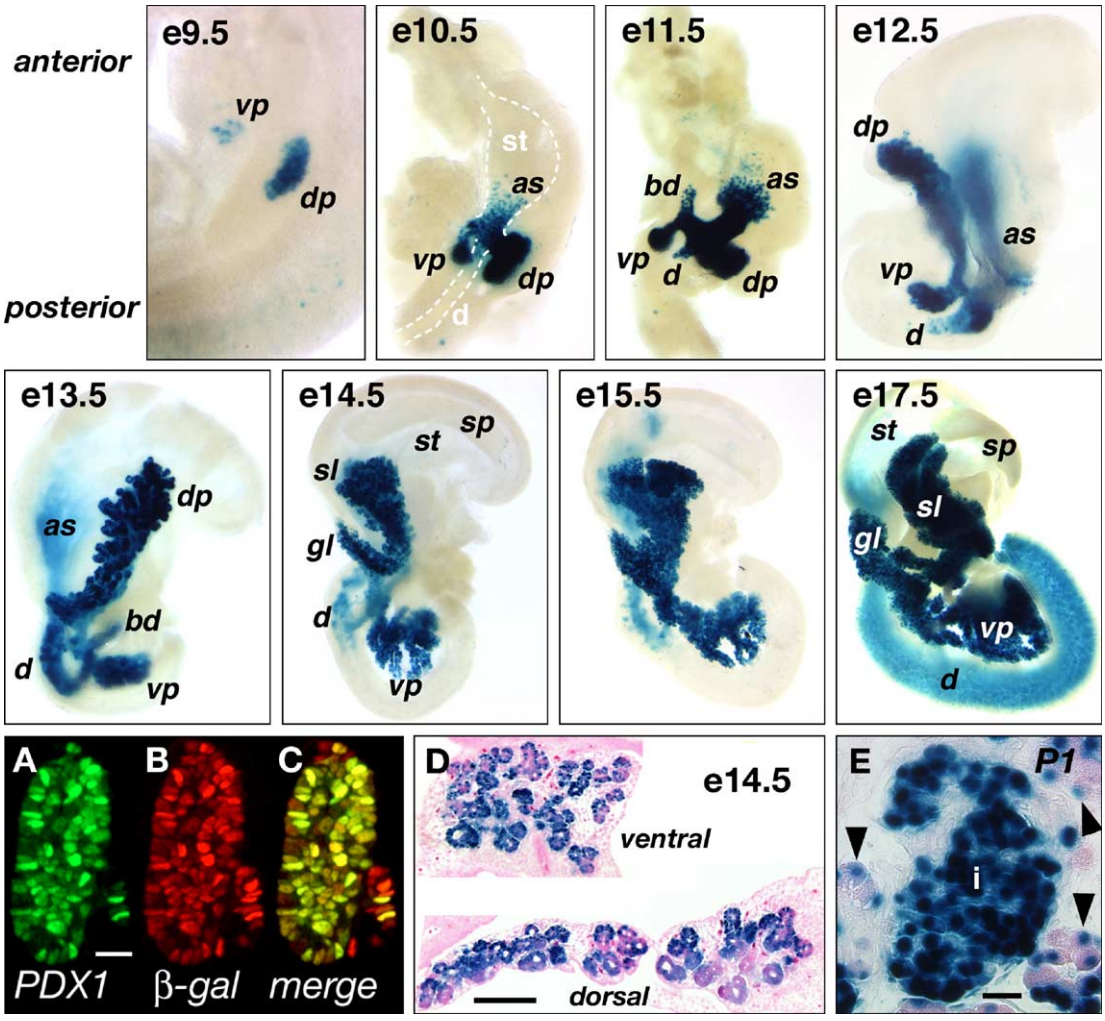

Fig. 3. Developmental expression of the tetO-Pdx1 transgene recapitulates normal *Pdx1* expression. Blue histochemical staining of β-galactosidase marks the sites of transgene activity for embryonic organs of the 958-1 line at E9.5 through E17.5. The gut tube for E10.5 is outlined. Gut structures are labeled as, antral stomach; bd, bile duct; d, duodenum; dp and vp, dorsal and ventral buds of the pancreas; gl and sl, gastric and splenic lobes of the pancreas; sp, spleen; st, stomach. (A–C) Immunofluorescent images of an E11.5 pancreatic epithelium for PDX1, β-galactosidase and the two merged, respectively. Scale bar = 20 μm. (D) Detection of transgenic β-galactosidase activity throughout the ventral and dorsal pancreatic epithelia from an E14.5 embryo. Scale bar = 200 μm. (E) β-galactosidase activity persists in both islet (i) and acinar (arrowheads) cells in postnatal day 1 pancreas. Scale bar = 20 μm.

levels of the transgenic and endogenous *Pdx1* mRNAs varied in concert during development (Fig. 4A), peaking during mid-development at E13.5–15.5.

Control of the tetO-Pdx1/nlacZ transgene

To verify that the nlacZ-form of the tTA-controlled transgene could be repressed in vivo, we administered the tetracycline analogue doxycycline to adult mice that had the tetO-Pdx1/nlacZ transgene and were heterozygous for $Pdx1^{tTA}$ (Fig. 4B). Prior to treatment with doxycycline, the transgenic Pdx1-nlacZ mRNA was measured from a biopsy sample of each pancreas. The transgenic mRNA was present only in those animals with both a $Pdx1^{tTA}$ allele and the transgene. After 7 days of recovery from the biopsy, doxycycline treatment was initiated for the same animals by an oral gavage and maintained with 1 mg/ml doxycycline in the drinking water. After 7 days of treatment, the level of the transgenic mRNA decreased at least 50-fold (Fig. 4B).

Doxycycline repression of pancreatic development

For embryos with the regulated genotype ($Pdx^{tTA/tTA}$; Tg^{Pdx1}), dox-treatment of the mothers from the morning of conception through birth prevented the development of a pancreas (compare Figs. 2A and E). Only very small epithelial remnants formed (Fig. 2F), which are characteristic of the germline *Pdx1*-knockout phenotype (Fig. 2B and Ahlgren et al., 1996; Offield et al., 1996). Initiating dox-treatment just prior to (E7.5) or at the onset of (E8.5) *Pdx1* expression produced similarly truncated epithelial remnants (Fig. 5). Thus, dox-treatment quickly and effectively blocked further pancreatic development.

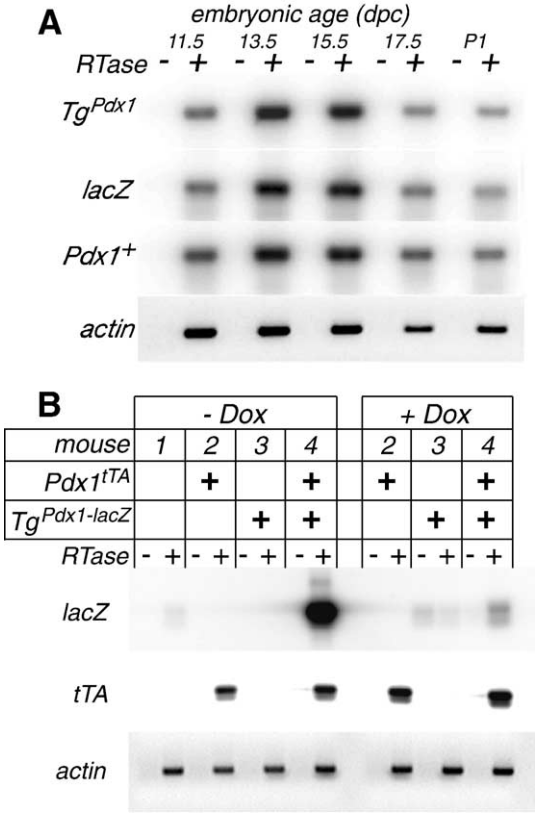

Fig. 4. Control of Pdx1 mRNA levels in embryonic and adult mice. (A) During embryonic development, the mRNA of the Pdx1 transgene profiles that of the endogenous *Pdx1* gene during embryonic development. RT-PCR analysis used gene-specific primers for the Pdx1 and nlacZ portions of the bicistronic transgenic mRNA, endogenous $Pdx1^{+}$ mRNA, and actin mRNA. Each PCR product was detected by Southern hybridization with a corresponding gene-specific oligonucleotide probe. The cDNA templates were derived from duplicate reactions without (−) and with (+) reverse transcriptase with RNA isolated from dissected pancreatic rudiments for E11.5 through postnatal day 1 (P1). (B) Doxycycline-treatment suppresses tTA-induced expression of the Pdx1-nlacZ transgene in adult mice. Prior to doxycycline-administration (*-Dox*), RNA was isolated from each of four adult mice representing normal (mouse 1), a single altered Pdx1 allele (mouse 2), a transgenic locus (mouse 3), and both modifications (mouse 4). Expression of the transgene (measured by amplification of the nlacZ cistron) requires tTA. After recovery, each mouse was administered doxycycline for 7 days and RNA was isolated from the remaining pancreas. Dox-treatment nearly eliminates the transgenic mRNA. RT-PCR analysis was performed and the products detected by Southern hybridization as described in Methods. Actin mRNA was measured to verify approximately equal assay conditions.

To test for *Pdx1* function at a specific developmental stage, repression of the Pdx1-transgene and turnover of the transgenic PDX1 protein must occur more quickly than the duration of the stage. Indeed, application of Dox to mothers on day 11.5 of gestation rendered *Pdx1* mRNA nearly undetectable in Dox-responsive embryos within 1 day (Fig. 6C) and the level of PDX1 protein was dramatically reduced (Fig. 6B). Epithelial morphogenesis diminished (compare panels 12.5 and +1d of Figs. 6A, B), indicating that PDX1 function became limiting well before 24 h of Dox-treatment. After 2 days, Pdx1 mRNA was more than 1000-fold lower than the normal E13.5 level, PDX1 protein was undetectable by immunohistochemistry, branching morphogenesis had stopped, and the epithelium formed abnormal, highly columnar ducts. These early effects of Dox were reproduced in three independent experiments. Thus, Dox-treatment initiated on E11.5 eliminates PDX1 prior to the beginning of the secondary transition at ~E13.5 and blocks further morphogenesis.

Beginning dox-treatment at later intervals resolved a progression of developmentally arrested pancreatic structures formed by the depletion of PDX1 at successively later developmental times (Fig. 5). Thus, doxycycline from E9.5 allowed growth of a linear epithelial tube with nascent invaginations that represent the beginning of primary branching. Doxycycline from E11.5 left a larger but crude duct-like structure with a few primary branches distally. Dox from E12.5 allowed the formation of extensive fine structure, which upon microscopic examination consisted of immature acini and associated small ductules (see below). The stepwise progression from a linear tube, to primary branches of large ducts, to secondary branches connecting acinar cell clusters (Fig. 5) defines the major morphogenetic changes of normal exocrine pancreatic development.

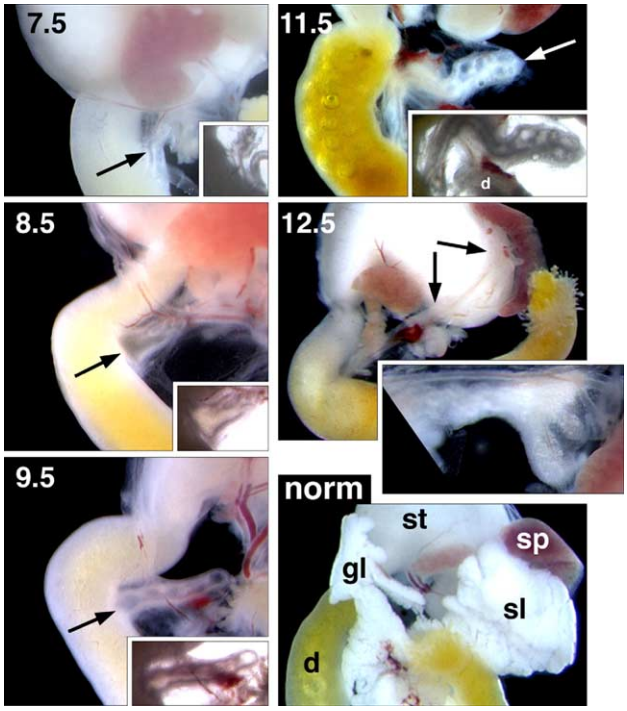

Fig. 5. Stepwise delayed doxycycline treatment resolves a progression of developmentally arrested structures. Dox-treatment was initiated by oral gavage at the days of gestation indicated and continued by inclusion in the drinking water. All images are of P1 pups. Arrows indicate pancreatic remnants. Insets are bright field images that show better the outlines of the pancreatic ductal epithelia. Normal pancreatic development is shown in the bottom right-most panel; this animal had the genotype $Pdx1^{+/tTA};Tg^{Pdx1\text{-}nlacZ}$. Abbreviations are the same as for Fig. 3.

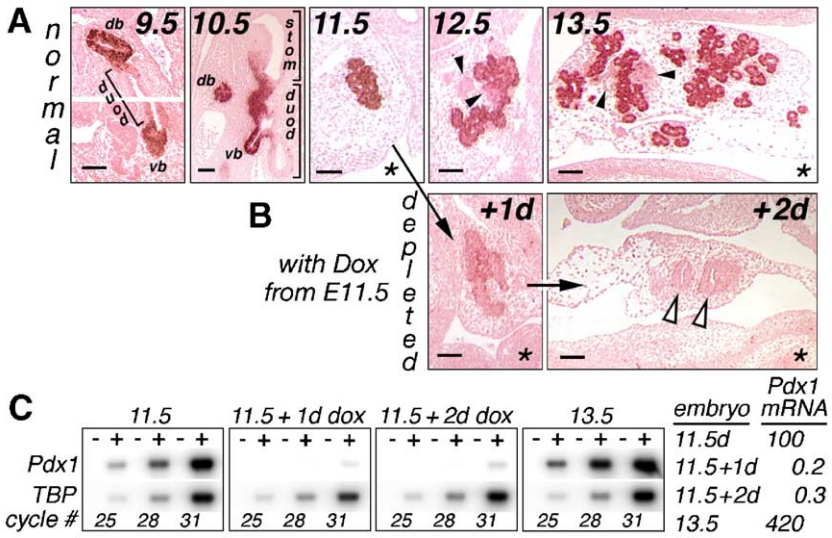

Fig. 6. Doxycycline-induced depletion of transgenic PDX1 is rapid. (A) Expression of the endogenous *Pdx1* gene is continuous. Immunohistochemical staining for PDX1 in genetically unaltered embryos (except E12.5d) is shown for E9.5 through E13.5. The E12.5 panel is from a $Pdx1^{+/rTA};Tg^{Pdx1}$ littermate of the Dox-responsive embryo of the +1d panel of part B; the presence of an unaltered *Pdx1* allele supports normal development in the presence of Dox. The epithelia of the stomach (stom), duodenum (duod) and the dorsal (db) and ventral (vb) pancreatic buds are indicated. The filled arrowheads point to clusters of early endocrine cells, which do not immunostain for PDX1. (B) Transgenic PDX1 in Dox-responsive embryos ($Pdx1^{rTA/rTA};Tg^{Pdx1}$) disappears during 2 days of continuous Dox-treatment. Dox was administered to pregnant females at E11.5 and embryonic visceral organs were obtained 1 and 2 days later. The morphological differences at E12.5 are representative of those observed for three independent experiments with separate litters. Sections were lightly counterstained with eosin. The asterisks mark the ages and treatments that were examined by RT-PCR in part C. Scale bars in panels of A and B = 100 μm. (C) The transgenic Pdx1 mRNA is nearly undetectable by RT-PCR after 1 day of Dox-administration. The amplification products are shown for 25, 28, and 31 amplification cycles and compared to the results for the TATA-binding protein (TBP) mRNA, which does not change in response to Dox. Hybridization signals were quantified by ImageQuant software from Phosphor-Images (Molecular Dynamics). Embryonic RNAs were isolated from dissected pancreatic rudiments and amplification templates from cDNA synthesis reactions without (−) and with (+) reverse transcriptase (RTase) were used to show the absence of a genomic DNA contribution to the PCR product.

The critical period of Pdx1 expression for acinar development

The most pronounced morphogenetic differences occurred between doxycycline initiation at E11.5 (PDX1-depletion by E12.5) and E12.5 (PDX1-depletion about E13.5). With PDX1-depletion at E12.5, dorsal and ventral remnants were present at birth (Fig. 7A); each was composed of a convoluted, partly branched duct-like epithelium of columnar cells encased in mesenchyme (Fig. 7B). Acinar-like cell clusters (Fig. 7B, open arrow) were rare (compare with Fig. 7H). A three-dimensional reconstruction of the epithelium shows an anastomosing duct-like network embraced by blood vessels (Fig. 8A). The distal tips of the duct-like remnant, which may have benefited from earlier and therefore longer *Pdx1* expression, have finer branches. The cells of the duct-like epithelium have a single cilium on the luminal plasmalemma (Fig. 8B), characteristic of both embryonic (Githens, 1989) and mature duct cells and distinct from acinar cells (Aughsteen, 2002). Although the epithelial structures look superficially like ducts, the cells are columnar rather than cuboidal (Fig. 7B, inset) and markers of ductal differentiation are either low (carbonic anyhdrase II; CAII) or absent (cytokeratin 7; CK7) (Fig. 7E). Prior to the onset of cellular differentiation, the epithelium of normal embryonic pancreas expresses glucose transporter 2 (GLUT2), whereas mature ducts and acini do not (Pang et al., 1994). The continued presence of GLUT2 in the duct-like epithelium at birth (Figs. 7C, D) also indicates that the premature depletion of PDX1 arrests ductal differentiation.

The block in acinar cell formation is reflected in the nearly complete loss of the mRNAs and proteins of amylase and carboxypeptidase A (CPA), digestive enzymes characteristic of differentiated acinar cells (Fig. 9). During normal development, amylase and CPA mRNAs increase logarithmically between E11.5 and E15.5 (Fig. 9 and Han et al., 1986) as the acinar lineage is defined and differentiation begins. PDX1-depletion from E12.5 decreases the accumulation of CPA and amylase mRNAs approximately 75- and 270-fold, respectively, by E15.5. The digestive enzyme mRNAs continue to rise for 1 day at a rate similar to that of normal development, then decrease after PDX1 falls below effective levels. The decrease reflects the nearly complete absence of acinar cell formation. The rare acinar-like cell clusters (Fig. 7B) present at birth contain low levels of CPA and amylase (Figs. 7F, G). These acini are continuous with the GLUT2-stained ductal epithelium, but do not stain for GLUT2 (Fig. 7), consistent with the expression of the acinar differentiation markers.

PTF1a is a bHLH transcription factor that becomes restricted to mature acinar cells and is the key transcriptional regulator of the digestive enzyme genes, including CPA and amylase (Cockell et al., 1989; Krapp et al., 1996; Rose et al., 2001). PDX1-depletion at E12.5 decreases Ptf1a mRNA about 90-fold, indicating that PDX1 is necessary for the nor-

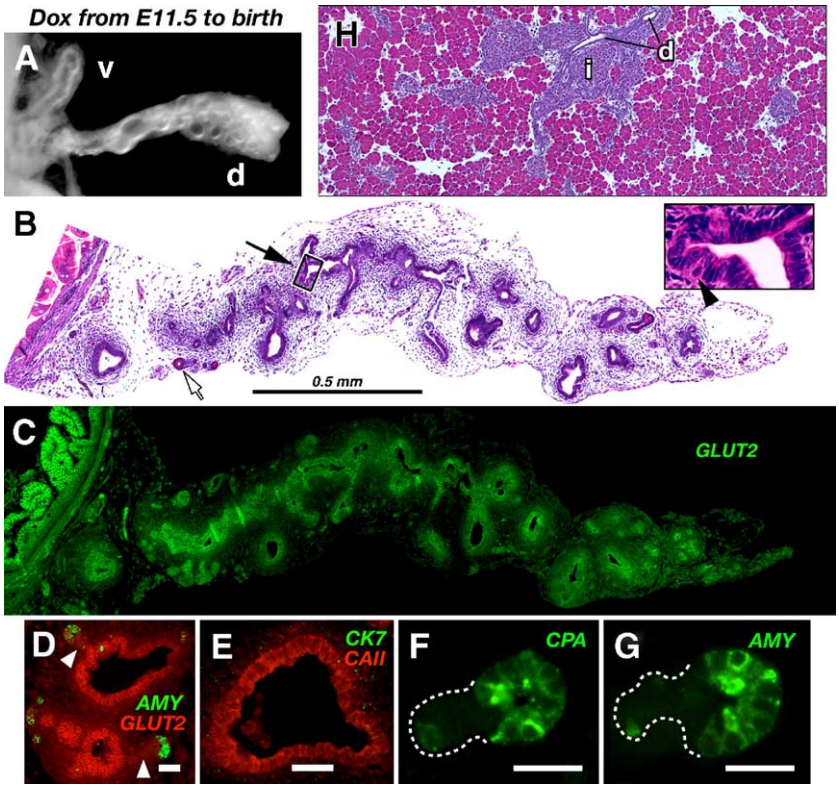

Fig. 7. The depletion of PDX1 at mid-pancreatic development blocks the transition from poorly branched, duct-like epithelium to highly branched ductules with immature acini. Pancreatic remnants are from neonates exposed to doxycycline from E11.5 to birth. (A) Whole remnant with epithelia derived from the dorsal (d) and ventral (v) pancreatic buds. (B) H&E-stained longitudinal section shows the highly columnar dorsal epithelium (inset) coursing through mesenchyme. The arrowhead indicates an isolated endocrine cell within the epithelial tube. Open arrow, a rare acinar structure; arrow, region enlarged in the inset. Scale bar = 0.5 mm, for panels B, C, and H. (C) The adjacent section was immunostained for GLUT2. (D) Amylase is present in small epithelial buds with diminished GLUT2; arrowheads point to epithelial bridges to the duct-like epithelium. (E) The epithelium has low carbonic anhydrase II (CAII) and no detectable cytokeratin 7 (CK7). The rare, immature acini co-express the acinar markers CPA (F) and amylase (G), from adjacent sections. Connections to the duct-like epithelium are outlined in white. The scale bars in panels D–G = 20 μm. (H) A small region of the extensive acinar tissue of a normal neonatal pancreas. i, forming islet; d, duct.

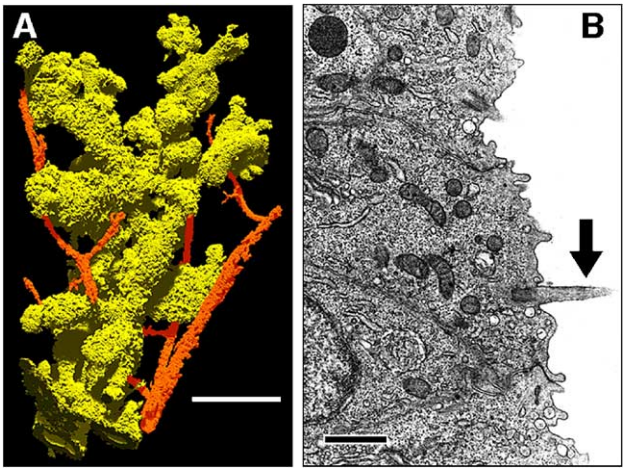

Fig. 8. Properties of the duct-like remnant from neonates treated with doxycycline from E11.5. (A) Three-dimensional reconstruction of the epithelium (yellow) shows a branched ductal structure with associated blood vessels (orange). Note that anastomoses are present. The sample was analyzed by Surface Imaging Microscopy by Resolution Sciences Corporation as described by Ewald et al. (2002). Scale bar = 200 μm. (B) The luminal surface of the duct-like epithelium has numerous small microvilli and a single cilium (arrow) for each cell; Scale bar = 2 μm.

mal increase of *Ptf1a* expression. In contrast to normal development, PTF1a is undetectable by immunofluorescence in the entire neonatal pancreatic remnant, including any immature acini, after dox treatment from E11.5 (data not shown).

To determine whether acinar cells form during PDX1-depletion and then disappear or simply never form, we examined pancreatic remnants from E14.5 embryos exposed to dox from E11.5. During normal development, nascent acini begin to appear at E13.5. At E14.5, morphologically distinct preacinar structures containing CPA are evident around the periphery of the pancreatic rudiment and endocrine cell clusters form in the gland center (Figs. 10A, B). Rudiments from dox-treated embryos do not produce preacini (Fig. 10C) and CPA-immunostained cells are rare (Fig. 10D), although endocrine cell clusters remain. Notch-signaling, which is required for proper pancreatic development (Apelqvist et al., 1999; Hald et al., 2003; Murtaugh et al., 2003), is disrupted: HES1, normally present in the tubular epithelium (Esni et al., 2004) is absent after dox-treatment (compare panels E and F of Fig. 10). The absence of preacini and the mRNAs for early acinar cell markers indicates that PDX1 is required to initiate the acinar lineage.

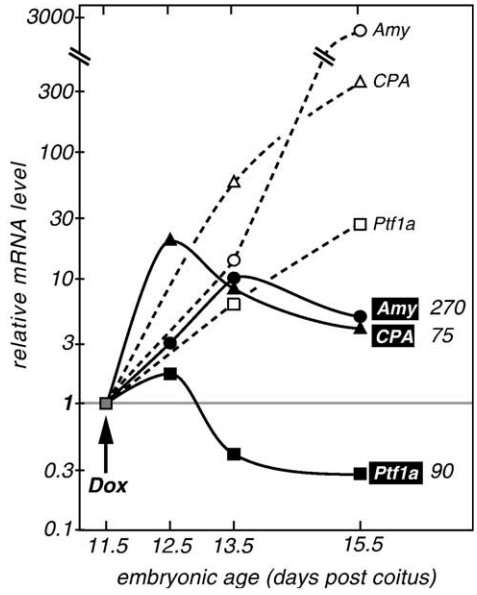

Fig. 9. Inhibition of the accumulation of acinar specific mRNAs by Dox-repression of *Pdx1*. mRNA levels were measured by quantitative RT-PCR (see Methods) with total RNA isolated from embryonic pancreatic rudiments dissected from embryos with and without maternal Dox-treatment beginning at 11.5 days of gestation. The level of each mRNA is expressed relative to its level at E11.5; note that the vertical axis is logarithmic. Dashed lines, without dox; solid lines, with dox. The fold-decreases of mRNA levels due to dox-treatment are indicated.

When dox-administration was begun 1 day later (E12.5), continued epithelial growth and branching created a larger remnant with a much smaller proportion of primitive duct (Figs. 11A, B). Most of the remaining duct-like tissue expressed GLUT2 and CAII, but not CK7 (Figs. 11C, D). Sparse CK7-staining indicates the formation of occasional regions of differentiated duct (Fig. 11D). The duct-like epithelium was largely replaced by numerous eosinophilic cell clusters that lacked the ductal markers CK7 and CAII (Figs. 11D–F). The cells of these clusters are polarized (extensive apical cytoplasm and basally located nuclei), arranged around a central lumen, and reminiscent of immature acini (Fig. 11b′). Compared to normal neonatal acini (Fig. 11a′), these have many more cells per acinus, less cytoplasm, and lower levels of the acinar markers CPA and amylase. In addition, numerous smaller ductules appear that connect directly to the immature acini (Fig. 11c′) and do not stain for GLUT2 (Figs. 11B, C). The appearance of ductules and acini indicates that three developmental processes were initiated prior to PDX1-depletion at approximately E13.5: new epithelial branching generating ductules that connect acini to the large ducts, acinar morphogenesis, and acinar cell differentiation. The presence of the immature acini at birth shows that once acinar differentiation has begun, PDX1 is not required to maintain these cells, but is required to complete their differentiation.

Discussion

Pdx1 is expressed in the developing pancreatic epithelium throughout mouse embryonic pancreatic development (Guz et al., 1995; Leonard et al., 1993). Germline inactivation of *Pdx1* demonstrated that it is necessary for the early phase of

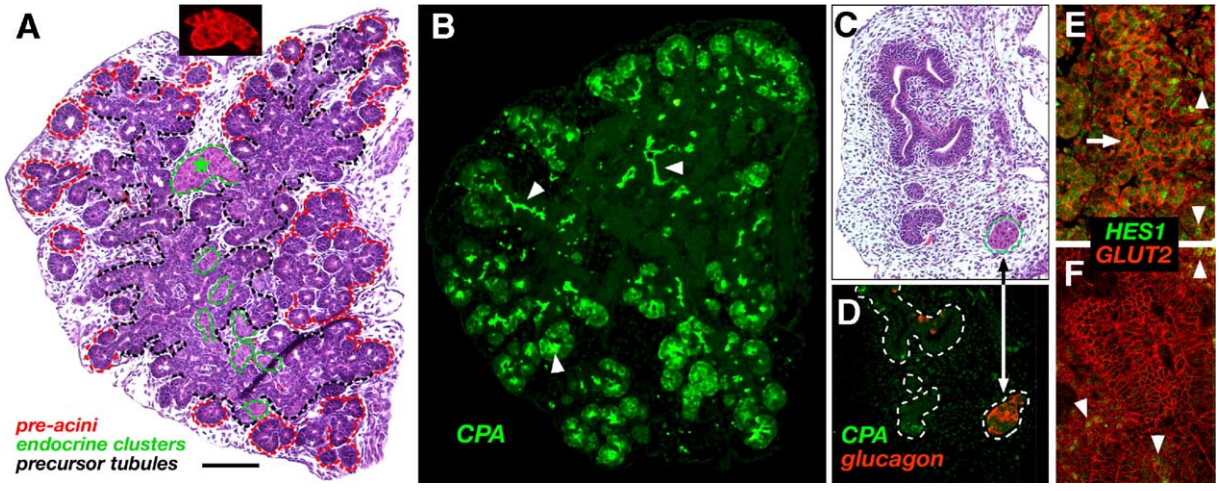

Fig. 10. Absence of PDX1 prevents the formation of acini rather than their maintenance. (A) H&E-stained section through the center of a normal mouse embryonic pancreas at E14.5. Recently formed preacini (red outlines) appear at the periphery, endocrine cell clusters (green outlines) accumulate in the interior, and the tubular precursor epithelial tree (black outline) extends from center to the peripheral preacini. Inset: immunofluorescent detection of glucagon in a nearby section for the same endocrine cluster indicated by the green asterisk. The scale bar represents 100 μm for three panels A–D. (B) Immunolocalization of CPA to peripheral cell clusters identifies the preacini in a section nearby that of panel A. Secreted CPA fills many ductal and acinar lumens (e.g., arrowheads). (C) A representative section of the much smaller remnant from an E14.5 embryo with regulated *Pdx1* after dox-treatment from E11.5. Epithelial mass is approximately 10% of normal. Recognizable preacinar structures are absent. A cluster of glucagon-expressing endocrine cells is retained (green outline). (D) Immunofluorescence of a section near to that of panel C shows the relative absence of CPA (compare panel B) and the presence of glucagon in the endocrine cluster continued from panel C. The epithelium is outlined in white. (E) In normal e14.5d pancreas, HES1 (green immunofluorescence) is present in nuclei of GLUT2-positive (red) epithelia (e.g., arrow) and mesenchyme (arrowheads). (F) After dox-treatment from e11.5, HES1 (green) is absent from the GLUT2-positive (red) epithelium but still present in the mesenchyme (arrowheads) at E14.5.

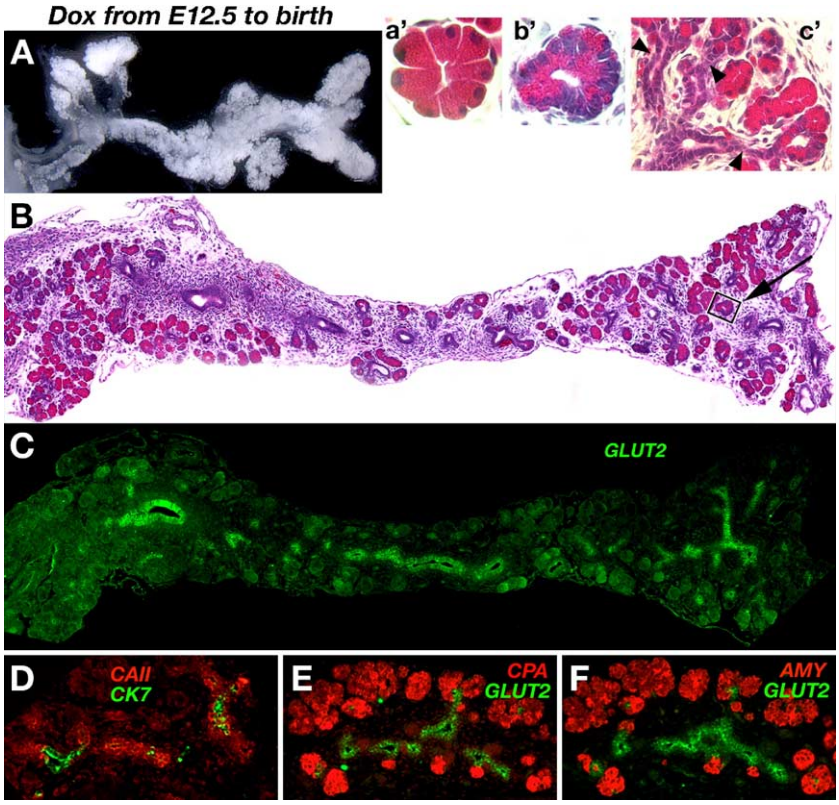

Fig. 11. The depletion of PDX1 by dox-treatment beginning E12.5 permits the transition to highly branched ductules with immature acini. (A) P1 remnant with acinar clusters. (B) H&E-stained longitudinal section shows large numbers of plump, eosinophilic, immature acini, and smaller amounts of columnar epithelium. The arrow indicates the acinus enlarged in b′. Insets: (b′) immature acinus with larger number of smaller cells with less cytoplasm compared to a normal P1 acinus (a′); (c′) narrow ductules (arrowheads) branching from a larger duct. (C) The remaining columnar duct expresses GLUT2. (D) The ductal tissue retains CAII and discrete sections now contain cytokeratin 7. The acinar structures contain both carboxypeptidase A (E) and amylase (F). Panels D, E, and F show similar regions of nearby, but nonadjacent, sections.

epithelial growth and branching morphogenesis (Jonsson et al., 1994; Ahlgren et al., 1996; Offield et al., 1996). We used a tetracycline regulatory scheme to show that PDX1 is subsequently required during mid-pancreatic development for cellular differentiation. Depletion of PDX1 after the early phase of pancreatic growth and morphogenesis blocks the next developmental phase, the onset of acinar and islet development. Our results prove that PDX1 is required for the initiation of the acinar lineage and not just for the elaboration of the precursor epithelium from which the acinar lineage derives. The efficacy of this experimental approach indicates that it can be generally useful to analyze temporally separate functions of regulators during an extended developmental process such as organogenesis. It provides a means to distinguish whether a developmental regulator is required before or during a developmental transition. In this instance, PDX1 is necessary during the formation of the epithelium from which the acinar cells arise and subsequently for the transition of the precursor epithelium to nascent acini.

The control of *Pdx1* function in vivo by doxycycline was sufficiently precise to resolve distinct stages of epithelial development. Administration of doxycycline to pregnant females at successively later intervals between E7.5 and E12.5 showed that PDX1 is necessary for growth and stepwise branching of the pancreatic epithelium. Dox-treatment early, at E7.5 or E8.5, prevented outgrowth of the pancreatic buds. Delaying application to E9.5 allowed the growth of a linear duct-like epithelium with invaginations at regular intervals that mark the sites for secondary branches. Dox-administration at E11.5 (with PDX1-depletion by E12.5) permitted further growth of the epithelium and the extension of the secondary branches. At birth, the PDX1-depleted remnants were approximately 10% normal size; consequently, PDX1 is required for growth as well as differentiation. Nonetheless, in the absence of PDX1, the ductal epithelium still grew approximately 10-fold from E15.5 to birth (data not shown), but the formation and growth of islet and acinar epithelia, which predominate late in pancreatic development, do not begin. Depletion of PDX1 shortly after the initiation of acinar development (dox at E12.5 with PDX1-depletion estimated at about E13.5) permits the formation of finer ducts and immature acini, but limits acinar growth and prevents complete differentiation.

Acinar development

Depletion of PDX1 at E12.5 halts epithelial differentiation just prior to the formation of acini. After 48 h of

depletion, the absence of preacini at E14.5, 1 day after they begin to appear during normal development, indicates that they do not initially form and disappear by birth. The epithelium appears blocked at an intermediate ductal phenotype with mixed properties of precursor epithelium (GLUT2 expression) and partial ductal differentiation (low CAII, but no CK7). Cells expressing acinar markers amylase and CPA were rare, and likely, the progeny of a few cells that had begun acinar differentiation just before PDX1 was depleted. This reproducible phenotype contrasts with that of pancreatic remnants when PDX1 was depleted a day later (E13.5). In this case, the delayed depletion of PDX1 allows the transformation of much of the duct-like epithelium to immature acini. The presence of these immature acini at birth suggests that after acinar fate has been specified, PDX1 is still required to complete the differentiation program. It is also possible that the delayed depletion of PDX1 at E13.5 allows the complete differentiation of this population early, but the level of differentiation is not maintained in the absence of PDX1. We consider this less likely, because PDX1 is not required to maintain the differentiated state of adult acinar cells (Holland et al., 2002).

The depletion of PDX1 prevents the accumulation of the mRNA for the PTF1a transcription factor. Because PTF1a is the acinar specific component of a bHLH factor complex that binds and activates the promoters of the digestive enzyme genes, it is likely that the lack of CPA and amylase gene products is due to the absence of PTF1a. The loss of PTF1a indicates that PDX1 is required either for *Ptf1a* transcription or for the formation of acinar precursor cells capable of activating *Ptf1a*.

Ductal development

The ductal system of the mature pancreas comprises the two main pancreatic ducts that drain into the intestine, small interlobular ducts that link the structural units of the acinar pancreas (lobules) to the main drainage, smaller intralobular ducts, and fine intercalated ducts that connect to acini (Ashizawa et al., 1997; Githens, 1989). The duct-like structure that forms after PDX1-depletion at E12.5 appears to represent incomplete main ducts (one from each bud), primary branches from the main ducts (the interlobular ducts), and the beginning of secondary branches distally (intralobular ducts). The large ducts are similar to and developmentally related to the common bile duct (Offield et al., 1996; Sumazaki et al., 2004). In the absence of PDX1 after E12.5, growth of the large ductal system continues until birth, but the small ducts, intercalated ducts, and acini do not form.

The coincidental absence of intercalated ducts and acini in the PDX1-depleted remnants is consistent with a concerted developmental program that embraces both cell types and is distinct from the program for the large ducts, which may be shared with the common bile duct. The program of large duct formation appears to separate from the acinar program at approximately E12.5 (Gu et al., 2002). Lineage tracing using the indelible Cre-lacZ genetic marker indicated that prior to E12.5 the precursor cells of the large ducts and acini share *Pdx1* expression. After E12.5, the acinar lineage continues *Pdx1* expression, whereas the ductal lineage does not. This resolution of the acinar and large-duct lineages at about E12.5 is consistent with our results. Depletion of PDX1 at E12.5 allows the formation of an extended tree of large ducts; but neither acini nor ductules form. However, PDX1-depletion about 1 day later allows the formation of acini and ductules similar to intercalated ducts.

In the mature gland, centroacinar cells are the transitional intercalated duct cells at the entrance of acini. Esni et al. (2004) have proposed that embryonic centroacinar cells demarcate the transition between the acinar precursor cells and the differentiating acinar cells in a continuous epithelium. Whereas the precursor cells have high levels of HES1 (generally thought to be an inhibitor of differentiation; Iso et al., 2003), the embryonic ‘centroacinar cells’ have lower levels of HES1 and no amylase, and the adjacent acinar cells have amylase, but no HES1. Thus, the embryonic centro-acinar cells may be the transitional cells in a concerted developmental process that gives rise to acini and associated intercalated ducts. The depletion of PDX1 at E12.5 disrupts proper Notch-signaling, so that epithelial *Hes1* expression is lost. PDX1 may be required, in part, to maintain Notch-signaling as a component of the acinar developmental decision process.

Acknowledgments

We thank M. Gossen, L. Henighausen, and H. Bujard for the tetracycline regulation constructs, Ping Lin for excellent technical assistance, Russell Kirschmann for assistance with the Resolve3D image analysis, B. Thorens for providing the GLUT2 antibody, T. Sudo for anti-HES1, Sherwood Githens for helpful discussions, and Galvin Swift for confocal imaging and many helpful criticisms of the manuscript. We thank C.V.E. Wright for providing the cloned mouse *Pdx1* gene and the unpublished information regarding its multiple transcriptional start sites and anti-PDX1 antibody. M.A.H. was a William K. Warren Diabetes Fellow. This work was supported by a Juvenile Diabetes International fellowship (to A.M.H.), an American Diabetes Association Mentor-based Postdoctoral Fellowship, and Research Grant DK-55266 from the National Institutes of Health.

References

Ahlgren, U., Jonsson, J., Edlund, H., 1996. The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. Development 122, 1409–1416.

Ahlgren, U., Pfaff, S.L., Jessell, T.M., Edlund, T., Edlund, H., 1997. Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature 385, 257–260.

Apelqvist, A., Li, H., L., S., Beatus, P., Anderson, D.J., Honjo, T., Hrabe de Angelis, M., Lendahl, U., Edlund, H., 1999. Notch signaling controls pancreatic cell differentiation. Nature 400, 877–881.

Ashizawa, N., Endoh, H., Hidaka, K., Watanabe, M., Fukumoto, S., 1997. Three-dimensional structure of the rat pancreatic duct in normal and inflammated pancreas. Microsc. Res. Tech. 37, 543–556.

Aughsteen, A.A., 2002. The ultrastructure of primary cilia in the endocrine and excretory ducts of mice and rats. Eur. J. Morphol. 39, 277–283.

Branda, C.S., Dymecki, S.M., 2004. Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice. Dev. Cell 6, 7–28.

Cockell, M., Stevenson, B.J., Strubin, M., Hagenbuchle, O., Wellauer, P.K., 1989. Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas. Mol. Cell. Biol. 9, 2464–2476.

Dutta, S., Bonner-Weir, S., Montminy, M., Wright, C., 1998. Regulatory factor linked to late-onset diabetes? Nature 392, 560.

Esni, F., Ghosh, B., Biankin, A.V., Lin, J.W., Albert, M.A., Yu, X., MacDonald, R.J., Civin, C.I., Real, F.X., Pack, M.A., Ball, D.W., Leach, S.D., 2004. Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. Development 131, 4213–4224.

Ewald, A.J., McBride, H., Reddington, M., Fraser, S.E., Kerschmann, R., 2002. Surface imaging microscopy, an automated method for visualizing whole embryo samples in three dimensions at high resolution. Dev. Dyn. 225, 369–375.

Furth, P.A., St.Onge, L., Boger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H., Hennighausen, L., 1994. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc. Natl. Acad. Sci. U. S. A. 91, 9302–9306.

Garrick, D., Fiering, S., Martin, D.I.K., Whitelaw, E., 1998. Repeat-induced gene silencing in mammals. Nat. Genet. 18, 56–59.

Githens, S., 1989. Development of duct cells. In: Lebenthal, E. (Ed.), Human Gastrointestinal Development. Raven Press, New York, pp. 669–683.

Gossen, M., Bujard, H., 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551.

Gu, G., Dubauskaite, J., Melton, D.A., 2002. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447–2457.

Guz, Y., Montminy, M.R., Stein, R., Leonard, J., Gamer, L.W., Wright, C.V.E., Teitelman, G., 1995. Expression of murine STF-1, a putative insulin gene transcription factor, in β cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. Development 121, 11–18.

Hald, J., Hjorth, P., German, M.S., Madsen, O.D., Serup, P., Jensen, J., 2003. Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. Dev. Biol. 260, 426–437.

Han, J.H., Rall, L., Rutter, W.J., 1986. Selective expression of rat pancreatic genes during embryonic development. Proc. Natl. Acad. Sci. U. S. A. 83, 110–114.

Holland, A.M., Hale, M.A., Kagami, H., Hammer, R.E., MacDonald, R.J., 2002. Experimental control of pancreatic development and maintenance. Proc. Natl. Acad. Sci. U. S. A. 99, 12236–12241.

Iso, T., Kedes, L., Hamamori, Y., 2003. HES and HERP families: multiple effectors of the Notch signal pathway. J. Cell. Physiol. 194, 237–255.

Jonsson, J., Carlsson, L., Edlund, T., Edlund, H., 1994. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371, 606–609.

Krapp, A., Knofler, M., Frutiger, F., Hughes, G.J., Hagenbuchle, O., Wellauer, P.K., 1996. The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix–loop–helix protein. EMBO J. 15, 4317–4329.

Leonard, J., Peers, B., Johnson, T., Ferreri, K., Lee, S., Montminy, M.R., 1993. Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. Mol. Endocrinol. 7, 1275–1283.

MacDonald, R.J., Przybyla, A.E., Rutter, W.J., 1977. Isolation and in vitro translation of the messenger RNA coding for pancreatic amylase. J. Biol. Chem. 252, 5522–5528.

MacDonald, R.J., Swift, G.H., Przybyla, A.E., Chirgwin, J.M., 1987. Isolation of RNA using guanidinium salts. Methods Enzymol. 152, 219–227.

Murtaugh, L.C., Stanger, B.Z., Kwan, D.M., Melton, D.A., 2003. Notch signaling controls multiple steps of pancreatic differentiation. Proc. Natl. Acad. Sci. U. S. A. 100, 14920–14925.

Offield, M.F., Jetton, J.L., Labosky, P.A., Ray, M., Stein, R.W., Magnuson, M.A., Hogan, B.L.M., Wright, C.V.E., 1996. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122, 983–995.

Ohlsson, H., Karlsson, K., Edlund, T., 1993. IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J. 12, 4251–4259.

Pang, K., Mukonoweshuro, C., Wong, G.G., 1994. Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas. Proc. Natl. Acad. Sci. U. S. A. 91, 9559–9563.

Pictet, R., Rutter, W.J., 1972. Development of the embryonic endocrine pancreas. In: Steiner, D.F., Freinkel, N. (Eds.), Handbook of Physiology. Section 7: Endocrinology, Endocr. Pancreas, vol. I. Williams and Wilkins, Baltimore, pp. 25–66.

Rose, S.D., Swift, G.H., Peyton, M.J., Hammer, R.E., MacDonald, R.J., 2001. The role of PTF1-P48 in pancreatic acinar gene expression. J. Biol. Chem. 276, 44018–44026.

Saez, E., No, D., West, A., Evans, R.M., 1997. Inducible gene expression in mammalian cells and transgenic mice. Curr. Opin. Biotechnol. 8, 608–616.

Schokett, P.E., Schatz, D.G., 1996. Diverse strategies for tetracycline regulated inducible gene expression. Proc. Natl. Acad. Sci. U. S. A. 93, 5173–5176.

Slack, J.M.W., 1995. Developmental biology of the pancreas. Development 121, 1569–1580.

Sumazaki, R., NShiojiri, N., Isoyama, S., Masu, M., Keino-Masu, K., Osawa, M., Nakauchi, H., Kageyama, R., Matsui, A., 2004. Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. Nat. Genet. 36, 83–87.

Zhu, Z., Zheng, T., Lee, C.G., Homer, R.J., Elias, J.A., 2002. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin. Cell Dev. Biol. 13, 121–128.